Journal of Bioenergetics and Biomembranes, Vol. 16, No. 3, 1984

Lack of Immunological Cross Reactivity Between the Transport Enzymes ($Na^+ + K^+$)-ATPase and ($K^+ + H^+$)-ATPase[1]

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Received September 23, 1983; revised October 31, 1983

Abstract

Goat antisera against ($Na^+ + K^+$)-ATPase and its isolated subunits and against ($K^+ + H^+$)-ATPase have been prepared in order to test for immune cross-reactivity between the two enzymes, whose catalytic subunits show great chemical similarity. None of the ($Na^+ + K^+$)-ATPase antisera cross-reacted with ($K^+ + H^+$)-ATPase or inhibited its enzyme activity. The same was true for the ($K^+ + H^+$)-ATPase antiserum with regard to ($Na^+ + K^+$)-ATPase and its subunits and its enzyme activity. So notwithstanding the chemical similarity of their subunits, there is no immunological cross-reactivity between these two plasma membrane ATPases.

Key Words: ($Na^+ + K^+$)-ATPase; ($K^+ + H^+$)-ATPase; goat antibodies; immunoblotting; immunological cross-reactivity; enzyme inhibition.

Introduction

The transport ATPases ($Na^+ + K^+$)-ATPase, which occurs in nearly all animal cells (Bonting, 1970), and ($K^+ + H^+$)-ATPase, which has only been detected so far in gastric parietal cells (Saccomani et al., 1979), have many characteristics in common (Schuurmans Stekhoven and Bonting, 1981). Both enzymes are located in the plasma membrane and are involved in the active transport of cations across that membrane, the energy for this process being delivered by the hydrolysis of ATP. They are intrinsic plasma membrane enzymes, which are lipid dependent. They have catalytic subunits with very

[1] Number LIII in the series *Studies on ($Na^+ + K^+$)-Activated ATPase.*

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0145-479X/84/0600-0223$03.50/0

similar molecular weights (Peters *et al.*, 1982), which can be phosphorylated by ATP. The amino acid composition of the two catalytic subunits is strikingly similar (Peters *et al.*, 1981, 1982) and probably also comparable to that of other plasma membrane ATPases (Kyte, 1981) like the $(Ca^{2+} + Mg^{2+})$-ATPase from erythrocytes (Graf *et al.*, 1982) and even to ATPases isolated from yeast (Foury *et al.*, 1981) and mold (Dame and Scarborough, 1981).

Recently, it has been shown that antisera against the subunits of $(Na^+ + K^+)$-ATPase from lamb kidney (Schellenberg *et al.*, 1981), guinea pig kidney (McDonough *et al.*, 1982), or shark rectal gland (Rhee and Hokin, 1975) each form immunocomplexes with the $(Na^+ + K^+)$-ATPase subunits of various organs and species. This implies that the $(Na^+ + K^+)$-ATPase of different species has common antigenic sites, indicating the conservative nature of this enzyme. The same is true for the plasma membrane $(Ca^{2+} + Mg^{2+})$-ATPase: antibodies directed to the enzyme from human erythrocytes cross-react with the enzyme from other species, e.g., from rabbit erythrocyte ghosts (Verma *et al.*, 1982). However, no cross-reactivity was observed with the $(Ca^{2+} + Mg^{2+})$-ATPase isolated from rabbit sarcoplasmic reticulum (Verma *et al.*, 1982). Neither was cross-reactivity found between an antiserum directed against guinea pig $(Na^+ + K^+)$-ATPase and the $(Ca^{2+} + Mg^{2+})$-ATPase from guinea pig sarcoplasmic reticulum (McDonough et al., 1982). This could mean that the common antigenic sites are restricted to ATPases of the plasma membrane. Since nothing is known about cross-reactivity between different plasma membrane ATPases, we have felt it to be interesting in the light of the above findings to study whether such an immunologic relationship exists.

In view of our experience with the purification of $(Na^+ + K^+)$-ATPase and $(K^+ + H^+)$-ATPase, we have chosen to study the immunologic relationships between these two plasma membrane ATPases.

Experimental

Enzyme Preparation and Assay

Highly purified membrane-bound $(Na^+ + K^+)$-ATPase and $(K^+ + H^+)$-ATPase preparations are obtained from rabbit kidney and porcine gastric mucosa, respectively, by means of the procedures described by Jørgensen (1974) and Schrijen *et al.* (1980).

$(Na^+ + K^+)$-ATPase activity is determined as described by Bonting (1970, p. 261), and $(K^+ + H^+)$-ATPase activity is assayed according to Schrijen *et al.* (1980).

For antisera inhibition assays, varying amounts of $(Na^+ + K^+)$-ATPase or $(K^+ + H^+)$-ATPase are preincubated in a total volume of 100 μl with

varying amounts of antiserum overnight at 4°C in a medium containing 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, and 25 mM imidazole-HCl (pH 7.4). In the case of ($K^+ + H^+$)-ATPase, NaCl is omitted. Aliquots (20 μl) of the preincubation mixture are used for the ATPase assay in a total volume of 400 μl. After stopping the ATPase reaction by adding 1.5 ml of a 8.6% (w/v) trichloroacetic acid solution, the protein precipitate is removed by centrifugation for 5 min at 6000 *g*. The amount of inorganic phosphate is then determined in 1.0 ml of the clear supernatant.

Antisera Production

Goat antisera are raised against the isolated, denatured α- and β-subunits of ($Na^+ + K^+$)-ATPase, against the fully active, highly purified ($Na^+ + K^+$)-ATPase, and against the isolated and denatured catalytic subunit of ($K^+ + H^+$)-ATPase. The isolated subunits for both enzymes are prepared as described previously (Peters *et al.*, 1981, 1982).

The following immunization procedure is used: goats are injected intracutaneously at multiple sites in the back with complete Freund's adjuvant, containing 0.5 mg ($Na^+ + K^+$)-ATPase holoenzyme, 0.5 mg isolated α-subunit, 0.27 mg isolated β-subunit, or 0.3 mg isolated catalytic subunit of ($K^+ + H^+$)-ATPase. Booster injections with equal amounts of protein in Freund's incomplete adjuvant are given subcutaneously at two different sites in the groin near a lymph node 3–4 weeks after the initial injection. These injections are repeated every 2 weeks in most cases (see Fig. 1).

When the titer begins to decrease, the goats are bled from the jugular artery. Control (preimmune) sera are obtained prior to injection of antigens.

Immunological Methods

The antiserum titer is determined by the microcomplement fixation test, which is performed as described by Margry *et al.* (1983) with some modifications: (1) Since no light-sensitive material is used, the whole procedure is carried out in daylight; (2) the antigens are not solubilized in detergent, but are the active membrane-bound ($Na^+ + K^+$)- and ($K^+ + H^+$)-ATPase preparations. The test is performed in a medium containing 5 mM sodium barbital, pH 7.4 (Merck AG, Darmstadt, FRG), 0.14 M NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.1% (w/v) bovine serum albumin (Fraction V, Sigma Chemicals, St. Louis, Missouri). The antiserum titer is defined as the reciprocal value of the antiserum dilution, which gives 50% of complement binding to the immune complex. Rabbit hemolysin and guinea pig complement (regular) are from Flow Laboratories, McLean, Virginia.

The immunologic specificity of the various antisera is examined by a modification of the blotting technique used by Towbin *et al.* (1979). The

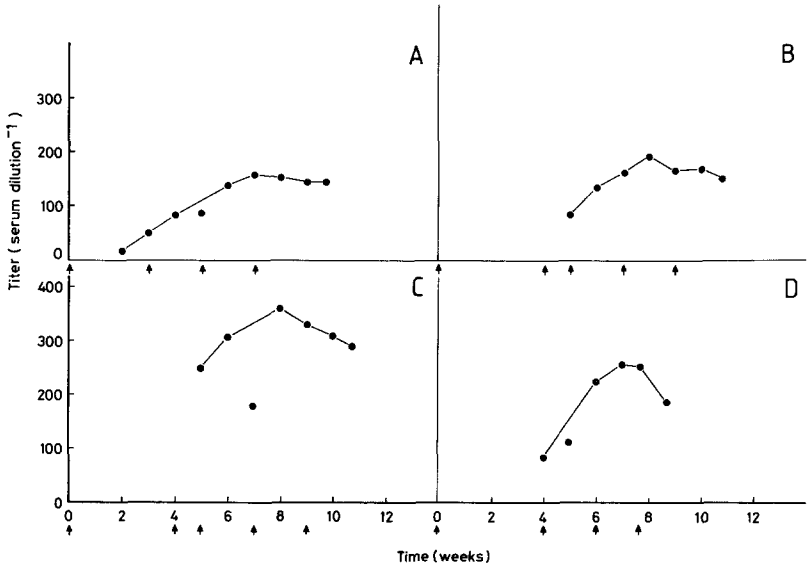

Fig. 1. Titer development in goats immunized with subunits and holoenzymes of (Na^+ + K^+)-ATPase and (K^+ + H^+)-ATPase. (A) denatured β-subunit of (Na^+ + K^+)-ATPase; (B) active, highly purified (Na^+ + K^+)-ATPase; (C) denatured α-subunit of (Na^+ + K^+)-ATPase; (D) denatured catalytic subunit of (K^+ + H^+)-ATPase. Arrows indicate when antigen injections are given.

proteins of (Na^+ + K^+)-ATPase or (K^+ + H^+)-ATPase, resolved by SDS polyacrylamide gel electrophoresis, are electrophoretically transferred (100 mA for 16 hr) to nitrocellulose paper (BA 85, Schleicher and Schüll, Dassel, FRG) as described by Vaessen *et al.* (1981). After the transfer, the nitrocellulose paper is incubated overnight with 10–100 times diluted antiserum. ^{125}I-Labeled protein A (Pharmacia, Uppsala, Sweden; specific activity 10 mCi/mg) is added, and autoradiography is carried out.

Results

The development of the goat antibodies directed against (Na^+ + K^+)-ATPase from rabbit kidney and (K^+ + H^+)-ATPase from pig gastric mucosa is followed by the microcomplement fixation test, using the corresponding active transport ATPase as antigen. Figure 1 shows the course of the antisera titers of the immunized goats. About eight weeks after the first injection the titers are maximal. The specificity of the antibodies is tested by the blotting technique described under Experimental.

Immune reactions of the various antisera with a highly purified (Na^+ +

K^+)-ATPase preparation are shown in Fig. 2. Slots 1 and 3 show the reaction of the antiserum with the denatured α-subunit, slot 4 that of the antiserum with the denatured β-subunit, and slot 5 that of the antiserum with the (Na^+ + K^+)-ATPase holoenzyme. The anti-α-subunit and anti-holoenzyme sera are directed only against the denatured α-subunit, and the former also to aggregates of the α-subunit. Although the anti-holoenzyme serum is raised against the nondenatured, fully active (Na^+ + K^+)-ATPase and thus may contain antibodies against the nondenatured β-subunit, it does not react with the denatured β-subunit. The β-subunit antiserum also contains antibodies against the α-subunit and its aggregates, but this is due to one unintentionally given α-subunit injection. The origin of the minor spots not due to the subunits of (Na^+ + K^+)-ATPase or its aggregates is not known.

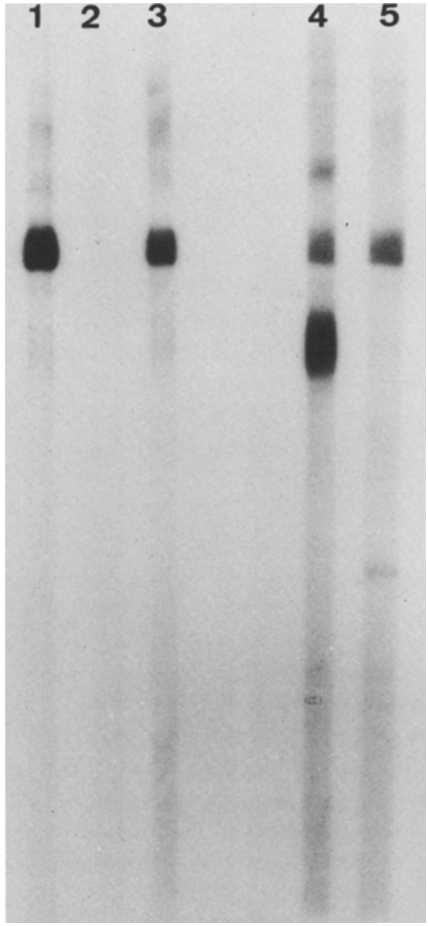

Fig. 2. Immune reactions of various antisera with highly purified (Na^+ + K^+)-ATPase. The enzyme (50 μg) is subjected to SDS gel electrophoresis and transferred to nitrocellulose, followed by incubation with antiserum. Bound antibodies are detected with (^{125}I)-labeled protein A, followed by autoradiography. Slots 1 + 3: antiserum raised against denatured α-subunit of (Na^+ + K^+)-ATPase; slot 2: antiserum raised against denatured catalytic subunit of (K^+ + H^+)-ATPase; slot 4: antiserum raised against denatured β-subunit of (Na^+ + K^+)-ATPase; slot 5: antiserum raised against (Na^+ + K^+)-ATPase holoenzyme.

Fig. 3. Immune reaction of antiserum with highly purified $(K^+ + H^+)$-ATPase. The enzyme (50 μg) is subjected to SDS gel electrophoresis and transferred to nitrocellulose paper, which is then incubated with the antiserum against the denatured catalytic subunit of $(K^+ + H^+)$-ATPase. Bound antibodies are detected by (^{125}I)-labeled protein A and autoradiography. The antisera against $(Na^+ + K^+)$-ATPase and its subunits do not react with the $(K^+ + H^+)$-ATPase (not shown).

Figure 3 shows the immunoreactivity of the antiserum, raised against the denatured catalytic subunit of $(K^+ + H^+)$-ATPase, with the highly purified $(K^+ + H^+)$-ATPase after gel electrophoresis and transfer to nitrocellulose paper. In addition to the large spot representing the $(K^+ + H^+)$-ATPase, there is a spot near the top of the gel, which is apparently due to an aggregate hardly entering the gel. Here a complication arises in the detection of goat antibodies by the binding of ^{125}I-labeled staphylococcal protein A. The antiserum of the goat immunized with denatured catalytic subunit of $(K^+ + H^+)$-ATPase (goat 37), when incubated with $(K^+ + H^+)$-ATPase on nitrocellulose paper, shows erratic results for its detection with ^{125}I-labeled protein A. This may be due to the more than 10,000-fold variation occurring in the binding of staphylococcal protein A to sera of different goats (Richman *et al.*,

1982). The immunoglobulins of goat 37 have most probably such a weak affinity for protein A that unequivocal detection becomes difficult. Hence, we have always performed simultaneous tests with (K^+ + H^+)-ATPase as antigen, when testing antiserum of goat 37 for cross-reactivity with (Na^+ + K^+)-ATPase.

The cross-reactivity of the anti(Na^+ + K^+)-ATPase and anti(K^+ + H^+)-ATPase sera has also been tested. In Fig. 2, slot 2 shows that the antiserum against the catalytic subunit of (K^+ + H^+)-ATPase does not cross-react with (Na^+ + K^+)-ATPase. Conversely, the antisera against the denatured α- and β-subunits and against (Na^+ + K^+)-ATPase holoenzyme do not react with (K^+ + H^+)-ATPase. These tests have been repeated several times with different nitrocellulose blots and different antisera dilutions, but cross-reactivity has never been detected.

In order to check the absence of cross-reactivity between (Na^+ + K^+)-ATPase and (K^+ + H^+)-ATPase, we have tested the effect of the antisera on ATPase activity. The (Na^+ + K^+)-ATPase activity is inhibited by the antisera against (Na^+ + K^+)-ATPase holoenzyme and against its α- and β-subunits (See Fig. 4). The anti-holoenzyme serum is the most potent in inhibiting the (Na^+ + K^+)-ATPase activity; a maximal inhibition of 85% is reached. The antisera against the α- and β-subunits cause a maximal inhibition of ca. 50%. The goat preimmune sera have no detectable effect on the ATPase activity. The antiserum against (K^+ + H^+)-ATPase does not

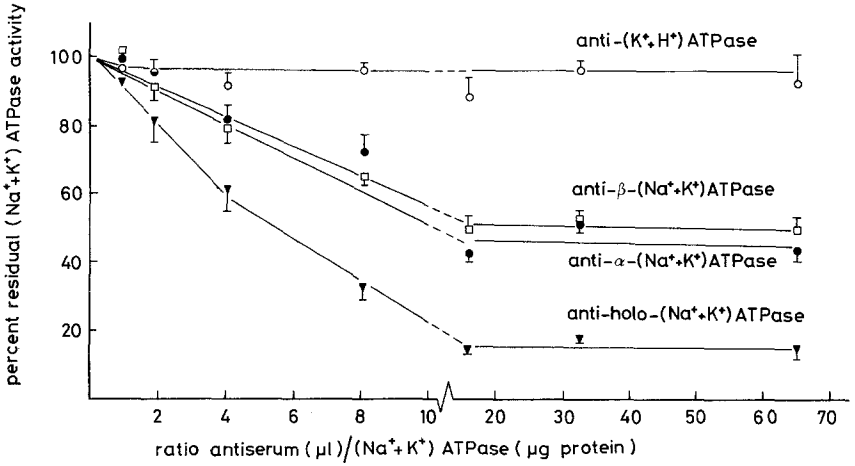

Fig. 4. Effect of different antisera on (Na^+ + K^+)-ATPase activity. (Na^+ + K^+)-ATPase is preincubated with antiserum, followed by assay of its activity as described under Experimental. Each point represents the mean of three different enzyme preparations; the bars indicate the standard error of the mean. (O) antiserum against (K^+ + H^+)-ATPase; (□) antiserum against β-subunit (Na^+ + K^+)-ATPase; (●) antiserum against α-subunit (Na^+ + K^+)-ATPase; (▼) antiserum against (Na^+ + K^+)-ATPase holoenzyme.

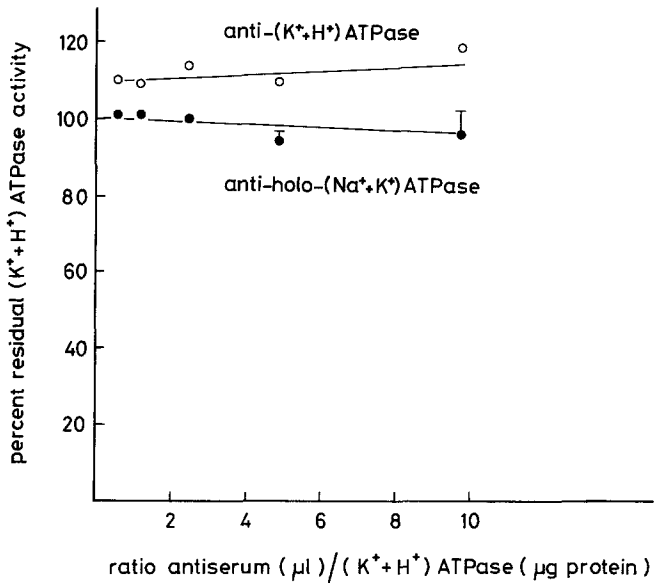

Fig. 5. Effect of different antisera on (K^+ + H^+)-ATPase activity. (K^+ + H^+)-ATPase is preincubated with antiserum followed by (K^+ + H^+)-ATPase activity determination as described under Experimental. Each point represents the mean of three different enzyme preparations; the bars indicate the standard error of the mean. (O) antiserum against the catalytic subunit of (K^+ + H^+)-ATPase; (●) antiserum against (Na^+ + K^+)-ATPase holo enzyme.

have any effect on the (Na^+ + K^+)-ATPase activity (Fig. 4). The (K^+ + H^+)-ATPase activity is neither inhibited by the antiserum against (K^+ + H^+)-ATPase, nor by the antiserum against the (Na^+ + K^+)-ATPase holoenzyme (Fig. 5).

Discussion

Although antisera are usually raised in rabbits, we have used the goat since our (Na^+ + K^+)-ATPase preparations are derived from rabbit tissue. A further advantage in using the goat is that large amounts of antisera of constant quality and characteristics can be obtained. There is not much difference in titer, whether the goat is immunized with SDS-denatured subunits or with active membrane-bound enzyme. All titer values are low, only slightly less than those reported by Girardet *et al.* (1981) for antisera against the α- and β-subunits of (Na^+ + K^+)-ATPase.

The specificity of the antisera has been unambiguously established by the

blotting technique. Both the antisera against α-subunit and against holo-$(Na^+ + K^+)$-ATPase react with the denatured α-subunit, but not with the denatured β-subunit. The antiserum against holo-$(Na^+ + K^+)$-ATPase might react with the nondenatured β-subunit, but this cannot be established with this technique.

All three $(Na^+ + K^+)$-ATPase antisera also react with the nondenatured α- and/or β-subunits, since they cause inhibition of ATPase activity. The antiserum against $(Na^+ + K^+)$-ATPase holoenzyme gives over 80% inhibition, while the α- and β-subunit antisera each give 50% inhibition. Comparable inhibition curves have been found by Zaheer *et al.* (1981) for dog kidney $(Na^+ + K^+)$-ATPase and its antibodies. As summarized by Lauf (1978), most $(Na^+ + K^+)$-ATPase antibodies are more or less inhibitory to enzyme activity, although recently McDonough *et al.* (1982) found no inhibition of the guinea pig enzyme by guinea pig kidney holoenzyme antibodies.

Our $(K^+ + H^+)$-ATPase antiserum does not inhibit the activity of the parent enzyme. Saccomani *et al.* (1979) have found such inhibition with antisera against pig gastric $(K^+ + H^+)$-ATPase from only two out of five immunized rabbits, although all five antisera showed precipitation lines with a $(K^+ + H^+)$-ATPase preparation in immunodiffusion and rocket immunoelectrophoresis.

Conventional (polyclonal) antisera raised against plasma membrane $(Na^+ + K^+)$-ATPase (Schellenberg *et al.*, 1981; McDonough *et al.*, 1982; Rhee and Hokin, 1975), $(Ca^{2+} + Mg^{2+})$-ATPase (Verma *et al.*, 1982), or $(K^+ + H^+)$-ATPase (Saccomani *et al.*, 1979) showed cross-reactivity with the corresponding transport ATPase of other species. However, Ball *et al.* (1982) have recently reported that a monoclonal antibody raised against lamb kidney $(Na^+ + K^+)$-ATPase does not cross-react with the enzyme from rat kidney, which may indicate that species-specific determinants are present and thus the structural similarity of the same plasma membrane ATPase from different species is not as pronounced as suggested by the results of polyclonal antisera. Plasma membrane $(Ca^{2+} + Mg^{2+})$-ATPase appears to have no common antigenic sites with the enzyme from sarcoplasmic reticulum (Verma *et al.*, 1982).

Recently, we have shown a striking resemblance in size and amino acid composition between the catalytic subunits of plasma membrane $(K^+ + H^+)$-ATPase and $(Na^+ + K^+)$-ATPase (Peters *et al.*, 1981, 1982). Nevertheless, we have now clearly established that despite this chemical resemblance there are little or no common antigenic sites exposed on the catalytic subunits of the two enzymes. The conclusion seems justified that with polyclonal antibodies cross-reactivity only occurs to the same plasma membrane transport ATPase of different species but not between different transport ATPases.

Acknowledgments

The authors wish to thank the personnel of the Animal Laboratory, College of Medicine, University of Nijmegen, for injecting and bleeding the animals and Mrs. Annie Fleuren-Jakobs for technical assistance.

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